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Effect of Phosphorylation on the Interaction between Sodium Channels and Ankyrin

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Voltage-dependent sodium channels(NaChs) are distributed at the nodes of Ranvier in myelinated axons which are critical for generation and propagation of neural signal[1]. The nodal distribution of NaChs is partly maintained by linkage between NaChs and axolemmal cytoskeletal protein, ankyrin[1]. To study how this linkage is regulated, we tested the effect of phosphorylation on their interaction because phosphorylation has been known to modulate interaction between proteins[2~5]. NaChs and ankyrin were purified from rat brain and human red blood cells, respectively. Ankyrin was iodinated and both NaChs and iodinated ankyrin were phosphorylated by cAMP-dependent protein kinase. The radioactivity of iodinated ankyrin bound to NaChs was measured. Phosphorylation of each protein decreased the interaction, and particularly, phosphorylation of ankyrin had a greater effect than that of NaChs. Phosphorylation of both proteins had an additive effect. These results suggest that phosphorylation may be involved in regulation of the interaction between NaChs and ankyrin.

Key Words: Ankyrin, Sodium channel, Phosphorylation

INTRODUCTION

NaChs mediate generation of action potentials by a rapid increase in Na^+ permeability[6]. In myelinated axons, NaChs are segregated and maintained at nodes of Ranvier. Recent studies have shown that this nodal distribution is induced by Schwann cells[1] and maintained by axonal cytoskeletal proteins[7]. Particularly, the ankyrin based-cytoskeleton is the most plausible basis for the organization of nodal cytoskeleton.

Ankyrin is a membrane-bound cytoskeletal protein and detected in red blood cells(RBCs), brain, and some other tissues. RBCs express two types of ankyrin isoforms while brain expresses more than four, and two of them are RBC-type[8,21]. Ankyrin from RBCs directly interacts with NaChs in a 1 : 1 ratio[9] and RBC-type isoform(s) of ankyrin are co-localized with NaChs at nodes of Ranvier[8]. Moreover, it was suggested that the linkage to ankyrin prevent free diffusion of NaChs in the membrane[7].

How is this linkage controlled for dynamic cell functions, such as metabolism of either NaChs or ankyrin? Protein phosphorylation is a general mechanism for regulating protein-protein interactions involved in several processes, such as neurotransmitter release[2], cell-substratum adhesion[3], and interaction between cytoskeletal proteins in erythrocytes[4,5]. Both NaChs and ankyrin are phosphoproteins[5,10,11] and phosphorylation of ankyrin alters the interaction with another cyteskeletal protein, spectrin[4]. Therefore, we hypothesized that phosphorylation of NaChs and ankyrin can regulate their interaction. The results of this study show that phosphorylation of either protein induced by cAMP-dependent protein kinase reduces their binding affinity. Phosphorylation of ankyrin had a greater effect than that of NaChs and phosphorylation of both proteins had an additive effect.

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MATERIALS AND METHODS

Purification of Ankyrin

Ankyrin was purified from one unit of human red blood cells (RBCs) as described[12]. RBCs were lysed to make ghost membrane from which crude ankyrin was extracted. Ankyrin was further purified using DE 52 (Whatman Biosystems) ion

exchange chromatography and a sucrose gradient. The purity of each fraction was assessed on a 4 to 15% gradient SDS-polyacrylamide gel[13] which was stained with silver nitrate[14]. Protein concentration was routinely determined by the method of Bradford[15].

Purification of NaChs

NaChs were purified from the brains of thirty male Sprague-Dawley rats[16]. Crude NaChs were extracted from synaptosomal membrane which was prepared from brain homogenate in the presence of 2.5% Triton X-100 and 0.25% phosphatidylcholine. Column chromatographies using DEAE-sephadex, hydroxyl apatite (Bio-Rad), and wheat germ agglutinin were carried out for further purification. Purified NaChs were reconstituted into unilamellar phospholipid vesicles[8].

Iodination of Ankyrin

Purified ankyrin was iodinated using ^{125}I-Bolton-Hunter reagent (ICN)[17]. Bolton-Hunter reagent was dried under nitrogen gas and then mixed with 10 μg of ankyrin in 100 μl of 50 mM phosphate buffer, pH 8.0, containing 0.05% Tween-20. After an hour, iodination reaction was stopped by the addition of 200 μg of unlabeled ankyrin. Unbound Bolton-Hunter reagent was removed from the mixture by microdialysis against 10 mM phosphate buffer, pH 7.4, containing 1 mM EDTA, 0.05% Tween-20, 1 mM NaN_3, and 10% sucrose. The specific activity of iodinated ankyrin was 7.8×10^6 cpm/mg protein.

Phosphorylation

NaChs reconstituted in liposome and ^{125}I-ankyrin were phosphorylated by cAMP-dependent kinase[5]. The reaction mixture contained 5~20 μg of either NaChs or ^{125}I-ankyrin, 0.2 mM ATP, 5 mM $MgCl_2$, and 2.5 units of kinase in 500 μl of 50 mM Tris, pH 7.5. After 90 minutes, kinase and free ATP were removed by a sephadex G-100 column prepared in a 3 ml syringe. The column was eluted with 50 mM Tris, pH 7.5. The fractions from the ankyrin column were then counted. The fractions which had high levels of radioactivity at the first peak were collected and used for the subsequent binding experiment. Phosphorylated NaChs were also collected from the NaCh column in the same range of fractions as the phosphorylated ankyrin was because the retention times for the two proteins were similar due to their similar molecular weights (220 kD vs. 260 kD)

Binding assay

Binding of phosphorylated or unphosphorylated NaChs to either phosphorylated or unphosphorylated ankyrin was examined as described[8]. One microgram of reconstituted NaChs was applied to each well of a blotting apparatus where nitrocellulose paper was installed at room temperature. One hour later, it was washed twice with 50 mM Tris, pH 8.0, containing 0.1% bovine serum albumin, 0.05% Triton X-100, and 0.0125% phosphatidylcholine. Various amounts (0, 10, 20, 40, 80, and 160 nM) of ankyrin were added to NaChs overnight at 4℃. Finally, it was washed three times as above. The nitrocellulose paper was exposed to X-omat RP X-ray film overnight and then, each well was cut out and the radioactivity was counted.

RESULTS

Purification of ankyrin and NaChs

Ankyrin was purified from one unit (450 ml) of human red blood cells(Fig. 1). Soluble cytoplasmic proteins, including hemoglobin, were removed by hypo-osmotic treatment leaving 200 ml of ghost membranes. The major membrane protein, spectrin (top two band marked by two arrowheads, molecular weight around 240 kDa in lane 2), was removed at the pre-extraction step, and another major protein, band 3 (broad band marked by an arrowhead, molecular weight around 97 kDa) was removed by DE 52 ion exchange column chromatography. Fractions from the sucrose gradient showed over 95% purity. Approximately, 2 mg of ankyrin was obtained. The molecular weight of purified ankyrin was 220 kD as measured by SDS-polyacrylamide gel electrophoresis (arrow in Fig. 1).

NaChs were purified from thirty rat brains (Fig. 2). Synaptic membranes were extracted by Triton X-100. NaChs from the WGA column showed greater than 90% purity. Approximately 200 μg of purified NaChs were obtained. The molecular weight of NaChs was 260 kD as measured by SDS-polyacrylamide gel electrophoresis (arrow in Fig. 2).

Effect of phosphorylation on interaction between NaChs and ankyrin

To investigate the effect of phosphorylation on interactions

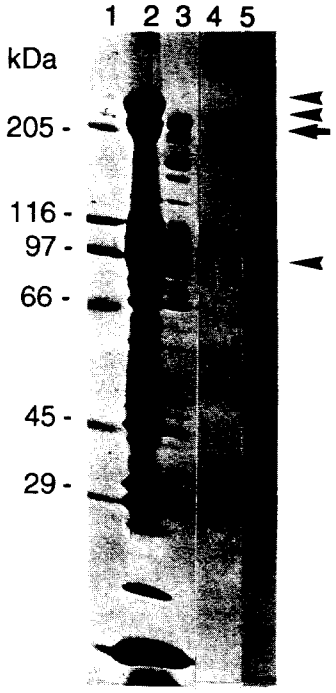

Fig. 1. Purification of ankyrin from erythrocytes. A 4~15% gradient SDS-polyacrylamide gel stained with silver nitrate shows the purity of ankyrin in each step. lane 1, molecular weight markers; lane 2, ghost membrane; lane 3, membrane extract; lane 4, DE-52 eluent; lane 5, purified ankyrin from sucrose gradient. Ankyrin, spectrin and band 3 are marked by top two arrow heads, an arrow and lower arrowhead, respectively.

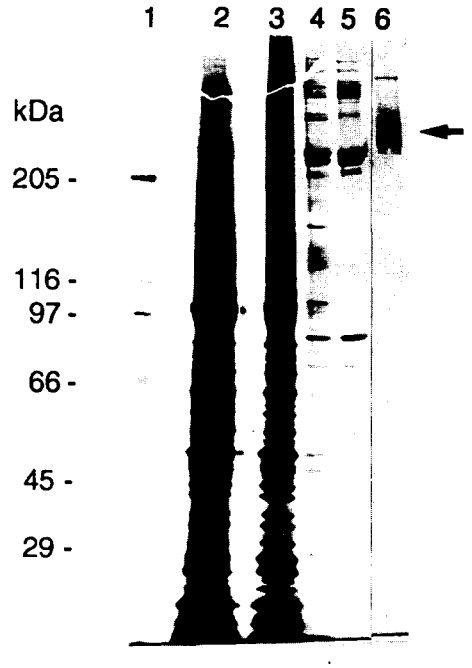

Fig. 2. Purification of NaChs from rat brains. A 4~15% gradient SDS-polyacrylamide gel was stained with silver nitrate. lane 1, molecular weight markers; lane 2, brain homogenate; lane 3, synaptosomal membrane; lane 4, DEAE-sephadex eluent; lane 5, HTP eluent; lane 6, WGA-column eluent. Nachs are marked by an arrow.

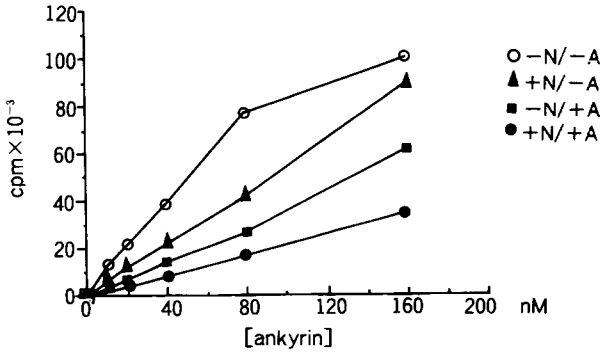

Fig. 3. Effect of phosphorylation on binding between NaChs and ankyrin. +, and - represent phosphorylated and unphosphorylated proteins, respectively. (o), binding between (−)-NaChs and (−)-ankyrin; (▲), (+)-NaChs and (−)-ankyrin; (■), (−)-NaChs and (+)-ankyrin; (●), (+)-NaChs and (+)-ankyrin.

between NaChs and ankyrin, binding experiments were carried out using a combination of phosphorylated or unphosphorylated NaChs and either phosphorylated or unphosphorylated ankyrin.

Phosphorylation of either NaChs or ankyrin decreased the interaction between two proteins (Fig. 3). Interestingly, phosphorylation of ankyrin had a greater effect than that of NaChs. This result suggests that phosphorylation could be involved in the regulation of the interactions between NaChs and ankyrin.

DISCUSSION

It has been shown that NaChs are linked to ankyrin[8] and that the linkage could be a constraint on mobility of NaChs in membranes[7,18]. The results from previous studies show that NaChs in cultured dorsal root ganglion cells are clustered on axons in the presence of Schwann cells[1]. Eighty percent of NaChs are linked to cytoskeletal proteins, possibly ankyrin,

and immobilized at early stages of development while 20% are not linked and mobile enough to make clusters[1,7]. In a demyelinating disease, such as multiple sclerosis, NaChs are diffused from nodes of Ranvier to internodal space[19]. In any case, the linkage between NaChs and ankyrin needs to be controlled for lateral diffusion of NaChs and/or for the metabolism of both NaChs and ankyrin.

Because protein phosphorylation has been considered as a general mechanism for regulating protein-protein interactions, phosphorylation of NaChs and ankyrin possibly controls their interaction. This study shows that phosphorylation of either NaChs or ankyrin decreases the affinity for each other. Thus, it is possible that phosphorylation of NaChs and ankyrin is increased during development and/or during pathological conditions which accompany changes in NaCh distribution. It also suggests that when a node of Ranvier is formed, cell adhesion molecules or extracellular matrix components produced by glial cells generate transmembrane signals which trigger dephosphorylation of either NaChs or ankyrin. This may result in stabilization of NaCh-ankyrin linkage and maintenance of nodal distribution of NaChs.

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